Animals
C57BL/KaLwRijHsd mice were purchased from Harlan CPB (Horst, The Netherlands). Male mice were 6 to 10 weeks old when used. Mice were housed and treated following the conditions approved by the Ethical Committee for Animal Experiments, VUB (license no. LA1230281). The animal ethics meet the standards required by the 1998 UKCCCR (United Kingdom Co-ordinating Committee on Cancer Research) Guidelines.

5T33vv multiple myeloma model
The 5T33MM cells originated spontaneously in aging C57BL/KaLwRij mice and have since been propagated in vivo by intravenous transfer of the diseased marrow in young syngeneic mice.1

Cells and cell culture conditions
Human myeloma cell lines RPMI-8226, Karpas-707, LP-1 and MMS1 were maintained in RPMI-1640 medium with 10% fetal calf serum (FCS) (Hyclone, UT, USA), mouse myeloma cell line 5T33vt was maintained in RPMI-1640 medium with 10% fetal bovine serum (FBS) (GIBCO, USA), supplemented glutamine (2mM), and antibiotics (penicillin 100 U/mL and streptomycin 50 µg/mL) (Lonzza, Belgium) at 37°C in a humidified 5% CO2 in-air atmosphere. Hypoxic (1%, 0% O2) conditions were established by culturing myeloma cells in a sealed chamber, 1% serum and 20mM HEPES (Sigma-Aldrich, St. Louis, USA) were supplemented in RPMI-1640 medium.

Cell cycle analysis
Cells were treated and analyzed after propidium iodide (PI) staining using standard methods. In brief, 1 ×10^6 cells per sample were washed once in cold phosphate buffer solution (PBS) and resuspended in 500 μL staining solution containing 50 μg/mL PI, 0.1% (vol/vol) Triton X-100, and 0.1% (wt/vol) sodium citrate. Cells were incubated at 4°C in the dark for 15 minutes and then analyzed by flow cytometry (Becton Dickinson, San Jose, CA, USA).

AnnexinV/PI staining
1 ×10^6 cells were washed twice with phosphate-buffered saline (PBS) and stained with 2 µl PI (50 μg/ml in PBS, BD Biosciences, San Jose, CA, USA) and 5 µl annexinV-FITC (BD Biosciences, San Jose, CA, USA) in 100 µl of binding buffer, and incubated at 4°C for 15 min. Then, cells were resuspended in 400 µl of binding buffer and immediately analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

Western blot
Cell pellets were lysed and protein extracts were blotted as previously described.2 The following antibodies were used: cyclin D1 (C-20), cyclin D2 (M-20), cyclin D3 (C-16), CDK2 (M-2), CDK4 (C-22), CDK6 (C-21), p21^{Cipl} (C-19), p27^{Kipl} (C-19), are all from Santa Cruz Biotechnology. Bax (#2772), Bcl-2 (#2870), Bcl-xL (#2764), Caspase-3 (#9665), Caspase-3 (#9665), Caspase-8 (#9746, #4927), Caspase-9 (#9502, #9504), PARP (#9542, #9544),
Phospho-Rb (Ser807/811) (#9308) were all purchased from Cell Signaling Technology (Danvers, MA, USA). HIF1α (#10006421) was from Cayman Chemical (Ann Arbor, Michigan, USA).

Assessment of oxygen tension in BM
To assess hypoxia, 6 naive mice and 6 5T33MMvv diseased mice were administered 60mg/kg Hypoxyprobe (pimonidazole, Hypoxyprobe Store, Burlington, MA, USA) intravenously (i.v.) 4 hours prior to taking a biopsy. Mouse tibia and femur were fixed in zinc fixative for 48 hours, decalcified for 48 hours, and embedded in paraffin. For detection of Hypoxyprobe, PAb2627 (rabbit polyclonal Ab, Hypoxyprobe Store, Burlington, MA, USA), secondary Ab (goat anti-rabbit IgG polymerized HRP, #3051-1, Epitomics, Burlingame, CA, USA), Universal Negative Control NC498AA, antigen retrieval reagent Pronase Reagent M31 and Betazoid DAB Chromogen Kit BDB2004H (Biocare Medical, Concord, CA, USA) were used. For endogenous hypoxia marker detection, HIF1α antibody was purchased from Novus Biologicals (Littleton, CO, USA).

In vivo analysis of tumor burden
Four groups of 10 mice were given intravenous injections of 5T33MMvv cells; one group of 10 naive mice was included as negative control. From the day of injection onward tumor-bearing mice were treated intraperitoneally with TH-302 (12.5 mg/kg, 25.0 mg/kg, 50.0 mg/kg) 5 times a week for 3 weeks, and the vehicle group mice received injections of saline. Liver and spleen were removed, weighed, and fixed. Blood samples were also obtained to determine serum paraprotein concentrations. Apoptosis was assessed by morphological assessment of HE stained BM sections.

Assessment of microvessel density
Microvessel density (MVD) was determined by CD31 staining as previously described. Briefly, mouse femurs were fixed in zinc fixative for 48 hours, decalcified for 48 hours, and embedded in paraffin. After blocking with normal goat serum, sections were incubated with a rat anti-CD31 antibody (PECAM-1, 1:10; PharMingen, San Diego, CA, USA) overnight at 4°C. A biotin-conjugated goat anti–rat antibody was used as secondary antibody (1:75 dilution, PharMingen, San Diego, CA, USA). A streptavidin-horseradish peroxidase conjugate in combination with tyramide signal amplification (TSA) (NEN Life Science, Boston, MA, USA) was used for detection. In the area with the highest blood vessel density (hot spot), the number of blood vessels was counted per 0.22 mm.

VEGFa ELISA
5T33vt MM cells were kept in 1% serum RPMI 1640 medium at a density of $1 \times 10^6$ cells/mL in normoxic (20% O2) and hypoxic (1% O2) conditions with or without 10 μM TH-302 for 24 hours. Conditioned media of the MM cells were analyzed with a VEGF ELISA kit according to manufacturer's instructions (Quantikine Mouse VEGF ELISA, R&D Systems, Minneapolis, MN, USA).
**Statistical analysis**
Statistical analysis was done using GraphPad Prism IV software. P values were calculated by Mann Whitney test. $p$ values $<0.05$ were considered significant. Mean ± SD is displayed in the figures.

**REFERENCES**

Figure S1. TH-302 induces Go/G1 cell cycle arrest in MM cells in a hypoxia selective manner. (A) Karpas-707 cell; (B) LP-1 cell; (C) MMS1 cell; (D) RPMI-8226 cell.
Figure S2. Western Blotting analysis of the mechanism of TH-302 induced G0/G1 cell cycle arrest
(A) Karpas-707 cell; (B) LP-1 cell; (C) MMS1 cell; (D) RPMI-8226 cell.
Figure S3. TH-302 triggers specific apoptosis in a dose-dependent manner in MM cells in hypoxic
(A) Karpas-707 cell; (B) MMS1 cell; (C) RPMI-8226 cell; (D) 5T33vt cell. *p<0.05, **p<0.01, ***p< 0.001, compared to 20% O₂ (n=3).
Figure S4. Western Blotting analysis of the mechanism of TH-302 induced MM cell apoptosis
(A) Karpas-707 cell; (B) MMS1 cell; (C) RPMI-8226 cell; (D) 5T33vt cell.
Figure S5. TH-302 decreases the accumulation of HIF1α in hypoxic MM cells (A) Karpas-707 cell; (B) RPMI-8226 cell; (C) 5T33vt cell.
Figure S6. The threshold of O$_2$ concentration for activating prodrug TH-302

MM cells were cultured in a series of sealed chambers filled with given concentration of oxygen for 24 hours. Effects of TH-302 on apoptosis was evaluated using flow cytometry as described in Materials and Methods. (A, B, C, D) individually represents the data in RPMI-8226, LP-1, MMS1 and 5T33vt MM cells. TH-302 was used at 5µM. *p<0.05, **p<0.01, significantly different from vehicle, n=3.
Figure S7. Evaluation of the safety of TH-302 in the naive mice

5T33vv mice were treated with vehicle or TH-302 at 50mg/kg for 3 weeks. (A) effects of TH-302 on body weight. (B) Effects of TH-302 on hemoglobin (HGB). (C) Effects of TH-302 on red blood cell count (RBC). (D) Effects of TH-302 on white blood cell count (WBC). (E) Effects of TH-302 on hematocrit (HCT). (F) Effects of TH-302 on microvessel density (MVD). *p* > 0.05, no significant difference between vehicle and TH-302 treated group, *n* = 3/group.